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Mitogenic Response of Mouse Spleen Cells and Gelation of Limulus Lysate by Lipopolysaccharide of *Yersinia pestis* and Evidence for Neutralization of the Lipopolysaccharide by Polymyxin B

THOMAS BUTLER[1]* AND GÖRAN MÖLLER[2]

Division of Geographic Medicine, Department of Medicine, Case Western Reserve University and University Hospitals, Cleveland, Ohio 44106,[1] and Division of Immunobiology, Wallenberg Laboratory, Karolinska Institute 104 05 Stockholm 50, Sweden[2]

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Lipopolysaccharide (LPS) extracted with phenol and water from *Yersinia pestis* was compared with LPS of *Escherichia coli* for stimulation of deoxyribonucleic acid synthesis in mouse spleen cells (lymphocyte mitogenesis), gelation of limulus lysate, pyrogenicity in the rabbit, and susceptibility to inhibition of these activities by polymyxin B sulfate (PBS). LPS of *Y. pestis* stimulated deoxyribonucleic acid synthesis in mouse spleen cell cultures over the same quantitative range as LPS of *E. coli*. In the limulus tests and rabbit pyrogenicity studies, the LPS of *Y. pestis* was active but about 10 times less potent than *E. coli* LPS on a weight basis. PBS in concentrations from 1 to 10 μg/ml diminished the rate of deoxyribonucleic acid synthesis in spleen cell cultures stimulated by LPS of both *Y. pestis* and *E. coli*. Addition of PBS to LPS of both *Y. pestis* and *E. coli* in a ratio of 100 parts of PBS to 1 part of LPS by weight increased by 10-fold the concentration of LPS required to produce gelation of limulus lysate and inhibited significantly pyrogenic responses in rabbits. These results demonstrating similarities of LPS of *Y. pestis* and *E. coli* may suggest that the pathogenesis of plague is similar to that of other gram-negative bacterial infections.

The causative bacterium of plague, *Yersinia pestis*, has been classified as a member of the family *Enterobacteriaceae* (32). The disease is characterized by fever, acute regional lymphadenitis, and bacteremia. Considerable evidence suggests that endotoxin plays a role in the pathogenesis of plague (5-8, 17). Previously described activities of lipopolysaccharide (LPS) of *Y. pestis* include lethality for mice (1, 14), pyrogenicity for rabbits (14), and local and generalized Shwartzman reactions (1).

More recently described activities of bacterial LPS that have not been examined in LPS of *Y. pestis* are murine B lymphocyte mitogenesis (2, 3, 10, 31), gelation of limulus lysate (15, 16, 21, 22), and susceptibility to inactivation by polymyxin B (11-13, 19, 25, 26, 28-30). The aims of the present study were to study these properties of LPS of *Y. pestis* comparatively with LPS of *E. coli* in order to gain a better understanding of the role of LPS in the pathogenesis of and immunity against plague infection.

MATERIALS AND METHODS

LPS extraction. An isolate of *Y. pestis* from a patient with plague in Vietnam in 1975 and *E. coli* O55:B5 were cultivated for extraction. Flasks containing tryptone-yeast-salt broth, prepared with pyrogen-free water, were inoculated with each bacterial strain and incubated at 37°C for 24 h. Formalin was added to make a 1% solution to kill the bacteria. The culture medium was centrifuged at 7,000 × *g* for 20 min. The sediment was washed in pyrogen-free saline and centrifuged, and the final sediment was lyophilized. LPS was extracted by the hot phenol-water method of Westphal et al. (36). The aqueous phase was washed five times with ether, dialyzed against distilled water, and lyophilized. The LPS of *E. coli* used in the pyrogenic studies and limulus tests was obtained from Difco Laboratories, Detroit, Mich. (lipopolysaccharide W, *E. coli* O127:B8).

Deoxyribonucleic acid (DNA) synthesis in spleen cell cultures. Single-cell suspensions from spleens of 8-week-old A × 5M female mice were prepared in serum-free Eagle minimum essential medium in Earle solution (supplemented with glutamine, nonessential amino acids, and pyruvate) containing 100 IU of penicillin and 100 μg of streptomycin per ml as described by Mishell and Dutton (24). With a Hamilton syringe, solutions containing LPS and polymyxin B sulfate (PBS; Minnesota 3M Laboratories, Leicestershire, England) in desired concentrations in medium were placed into flat-bottom wells of tissue culture plates (Falcon Plastics, Oxnard, Calif.). Mononu-

clear cells (4×10^5) were then added to each well. Cultures were incubated at 37°C in an incubator with a gas composition of 10% CO_2, 7% O_2, and 83% N_2. After 24 h, 0.05 ml of [*methyl*-^{3}H]thymidine (1 μCi; specific activity, 5 Ci/mmol; Radiochemical Centre, Amersham, England) was added to each culture. After an additional 24 h, the culture plates were harvested with a multiple automated sample harvester (Skatron, AS, Lierbyen, Norway). The glass filter pads were dried and placed in vials containing 5 ml of scintillation fluid (5.5 g of Permablend III [Packard Instrument Co., Downers Grove, Ill.] in 1 liter of toluene). The vials were counted in a Tri-Carb liquid scintillation spectrometer (Packard). Means of counts per minute and the standard errors of triplicate cultures were computed.

Limulus assay. Limulus amebocyte lysate was obtained from the laboratory of J. Levin, and the test was performed as previously described (21, 22). Equal volumes of limulus lysate and test solution in 0.9% sodium chloride were incubated at 37°C for 3 h and allowed to stand at room temperature for 18 h. A gel or flocculation in the tube indicated a positive result. The limulus test detected as little as 0.1 ng of *E. coli* LPS standard per ml.

Pyrogenic responses. New Zealand white rabbits weighing 2 to 3 kg were trained in loosely fitting wooden stocks the day before the experiment for 4 h. Rectal temperatures were measured hourly by thermistors connected to a telethermometer (Yellow Springs Instrument Co., Yellow Springs, Ohio). Animals with temperatures outside the range of 38.5 to 39.9°C on the day before or on the day of the experiment were excluded. Test solutions in 0.5 ml of pyrogen-free 0.9% sodium chloride were injected into the marginal vein of the ear. Hourly temperature over a 5-h period were plotted on 1-by-1-inch (ca 2.5 by 2.5 cm) graph paper, with 1°C and 1 h equaling 1 inch (ca. 2.5 cm). The fever index was measured with a compensating polar planimeter (Keuffel and Esser Co., Germany) as the area under the curve above the initial temperature base line over a 5-h period (20, 37). In the pyrogenic studies and limulus tests, PBS (Aerosporin, Burroughs Wellcome, Research Triangle Park, N.C.) was combined with LPS in solution in a weight ratio of 100 parts of PBS to 1 part of LPS and incubated at 37°C for 15 min.

RESULTS

DNA synthesis in spleen cell cultures. A mitogenic response in spleen cell cultures stimulated by LPS was demonstrated by enhanced rates of DNA synthesis. LPS from both *E. coli* and *Y. pestis* over a dose range from 0.1 to 100 μg/ml caused linear increases in DNA synthesis (Fig. 1). Both LPS preparations were about equally potent.

Limulus tests. The least concentrations of LPS that produced a positive limulus test were 0.0001 μg/ml for *E. coli* and 0.001 μg/ml for *Y. pestis* (Table 1). The potency of the LPS of *Y. pestis* was thus about 10 times less than the LPS of *E. coli* in the limulus test.

Pyrogenic studies. The LPS extracted from

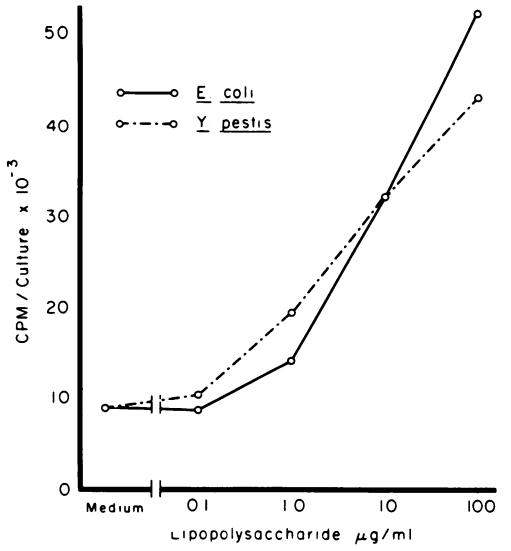

FIG. 1. *DNA synthesis in murine spleen cell cultures stimulated by LPS in doses from 0 to 100 μg/ml. Mean counts per minute are shown in triplicate cultures after 48 h of incubation with LPS and after 24 h of incubation with tritiated thymidine.*

TABLE 1. *Reaction of limulus lysate with LPS and inhibition of the reaction by PBS*[a]

Concn of LPS (μg/ml)	Results of limulus test[b]			
	E. coli		*Y. pestis*	
	No PBS	PBS	No PBS	PBS
0.01	+	+	+	+
0.001	+	+	+	−
0.0001	+	−	−	−
0.00001	−	−	−	−

[a] All solutions with PBS contained a weight ratio of 100 parts of PBS to 1 part of LPS.

[b] Symbols: +, Gel or flocculation; −, negative.

Y. pestis was pyrogenic for rabbits. The dose response curves revealed that the LPS of *Y. pestis* was approximately 10 times less pyrogenic on a weight basis than the LPS of *E. coli* (Fig. 2). Both LPS preparations showed a linear response over a dose range from 0.01 to 1.0 μg.

Inhibition of LPS activities by PBS. The effect of the concentration of PBS in spleen cell cultures was studied. Concentrations between 1 and 10 μg/ml reduced the rate of DNA synthesis in the presence of LPS. With concentrations of PBS greater than 10 μg/ml, unstimulated cultures showed reductions in counts per minute, and cell viability was diminished, as shown by trypan blue dye exclusion after 48 h in culture. The effect of PBS in a concentration of 4 μg/ml in the presence of 10 μg LPS per ml is shown in Fig. 3. The mitogenic activities of LPS of both

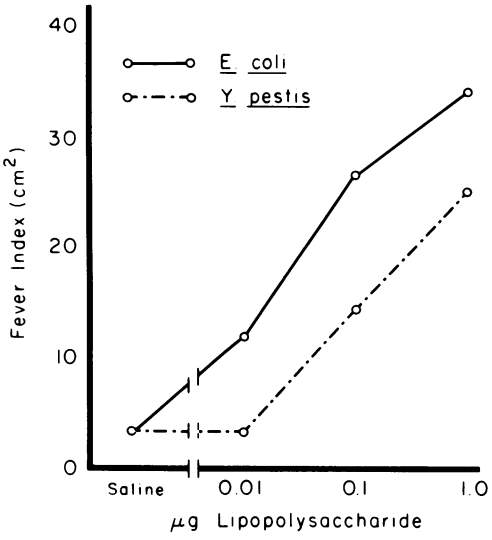

FIG. 2. *Pyrogenic responses of rabbits to intravenously injected LPS in 0.5 ml of normal saline. Fever index is the area under the temperature curve above the starting base line for 5 h after the injection. Each point is the mean of four or more rabbits.*

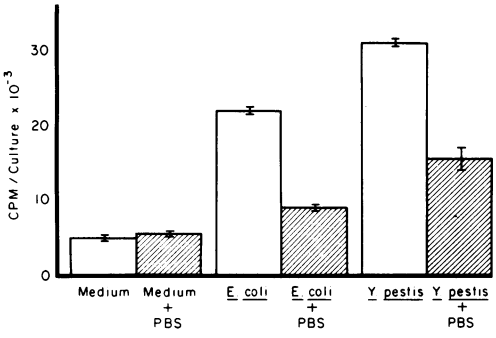

FIG. 3. *Inhibition of LPS-stimulated DNA synthesis by PBS. Open bars are the means of triplicate cultures with standard error of the mean unstimulated (medium) and stimulated by 10 μg of LPS of E. coli or Y. pestis per ml. Cross-hatched bars are means of triplicate cultures containing PBS in a concentration of 4 μg/ml.*

Y. pestis and *E. coli* were significantly inhibited by PBS ($P < 0.05$ by Student's t test).

Addition of PBS to LPS in a ratio of 100 parts of PBS to 1 part of LPS by weight before incubation with the limulus lysate resulted in a 10-fold loss of LPS activity for both LPS preparations (Table 1). The 10-fold loss in LPS activity in the limulus test by the addition of PBS was reproducible in three experiments. Similarly, 100 parts of PBS added to 1 part of LPS by weight followed by 15 min of incubation at 37°C resulted in diminished pyrogenic responses (Fig. 4). The differences between the responses

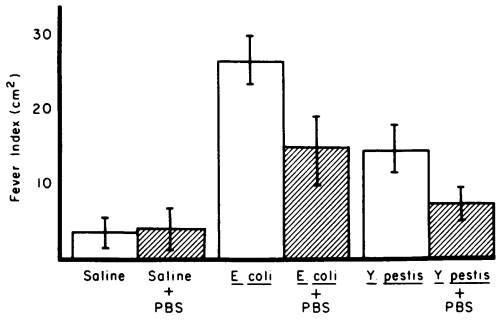

FIG. 4. *Inhibition of LPS-induced pyrogenic responses in rabbits by PBS. Open bars are the means of 5-h fever indexes with standard errors of the means in five rabbits injected with saline or 0.1 μg of LPS of E. coli or Y. pestis. Cross-hatched bars show means of fever indexes in five rabbits injected with 10 μg of PBS (100 parts of PBS to 1 part of LPS by weight incubated together for 15 min at 37°C before injection).*

to LPS alone and to LPS with PBS were statistically significant ($P < 0.05$) for both LPS preparations by Student's t test.

DISCUSSION

This is the first report that LPS of *Y. pestis* is mitogenic for spleen cells and reacts with limulus lysate. These two activities, as well as pyrogenicity in the rabbit, were inhibited by the addition of polymyxin B to solutions of LPS from both *Y. pestis* and *E. coli*. These findings, when combined with the previously known activities of LPS of *Y. pestis*, including lethality for mice, pyrogenicity in the rabbit, and the production of local and generalized Shwartzman reactions (1, 14, 34, 35), demonstrate additional similarities of the biological properties of the LPS of *Y. pestis* and the endotoxins of other gram-negative bacteria.

The mitogenic activity of LPS of *Y. pestis* in spleen cell cultures may be relevant to the pathogenesis and immunity of plague infections. LPS of other bacteria has been shown to be selectively mitogenic for B lymphocytes (2, 3, 10, 27, 31). In plague infection, bacteria proliferate within mononuclear phagocytes of the lymph node during the incubation period (9). Subsequently, when the lymph node enlarges, large numbers of bacteria are present in close proximity to germinal centers containing B lymphocytes (18). Another role of B lymphocyte mitogenesis may be in defending the host against the infection through the production of antibodies. In the C3H/Hej mouse, a genetic inability of B lymphocytes to respond to LPS is associated with a diminished resistance to intraperitoneal infection with *Salmonella typhimurium*

(33), indicating that B lymphocyte mitogenesis may be a mechanism for defending the host against bacterial infection.

The susceptibility of LPS of *Y. pestis* to inhibition by polymyxin B establishes an additional similarity of the LPS to that of other bacterial species (11–13, 19, 25, 26, 29, 30). The mechanism of polymyxin B inhibition may be a physical or chemical alteration of the LPS molecule (4, 23), which is the result of binding of the cationic polymyxin molecules to negatively charged groups in the polyanionic LPS. Another possible mechanism for the inhibition of lymphocyte mitogenesis is a direct toxicity of polymyxin B for lymphocytes. Studies of other mitogens, including PPD, PHA, and concanavalin A, revealed, however, no inhibitory action of polymyxin B for these other lymphocyte mitogens in spleen cell cultures (19).

Attempts have been made to exploit the anti-LPS activity of polymyxins for therapeutic advantage in gram-negative infections. Unfortunately, high toxic doses were required to achieve the anti-LPS activity in dogs (13). Nevertheless, polymyxin B may prove to be a useful tool for probing the functions of LPS and for modifying disease in experimental gram-negative bacterial infections.

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